EDEMA FACTOR AND PHOSPHOLIPASE RELEASE BY A STRAIN OF BACILLUS CEREUS

ROBERT A. ALTENBERN AND HAROLD B. STULL
U.S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland

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U.S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland

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Abstract

After ultraviolet light irradiation, strain 6464 of *Bacillus cereus* lysed, resulting in the release of toxin, phospholipase, and mature phage particles. Small amounts of toxin and phospholipase produced by non-induced cultures were correlated with the infrequent spontaneous release of bacteriophage. Stationary incubation following ultraviolet induction results in a greater yield of toxin and phospholipase than post irradiation incubation on a shaker. Post-irradiation incubation at temperatures below 37° either reduced (30°) or prevented (26°) toxin and phospholipase production. A clone was obtained which was sensitive to the phage from the parent strain and was presumably no longer lysogenic for it. This cured strain still exhibited ultraviolet-induced optical density decline accompanied by release of toxin and phospholipase. Mitomycin C would induce strain 6464 and the cured strain derived from it and both released toxin and phospholipase during mitomycin C induced lysis. The induced lysis of the cured strain could be prevented by postinduction treatment with inhibitors of synthesis of protein (chloramphenicol), RNA (actinomycin D), or DNA (5-fluorouracil deoxyriboside).

Introduction

Few previous studies have investigated the possibility that production of toxins and phospholipase by strains of *Bacillus cereus* was other than a consequence of normal bacterial growth. The paper by Kushner (4) clearly showed that under some conditions, phospholipase was produced during logarithmic growth whereas other growth conditions allowed lecithinase production only after the logarithmic growth phase. A preliminary report from this laboratory (1) has shown that there is a pronounced liberation of phospholipase, phosphatasemia factor (7), and edema factor following ultraviolet induction of a lysogenic strain of *B. cereus*. This paper describes additional experiments concerning this phenomenon and the results indicate that the liberation of these entities is associated with the bacterial lysis which follows treatment with agents which typically induce the development of temperate bacteriophage.

Methods

The strains of *B. cereus* employed were those from the stock cultures at this laboratory. Spore suspensions were prepared from potato agar slants for each one of these strains according to the method of Thorne (10). Unless otherwise noted, the growth medium was the casamino acids medium described by Thorne and Belton (8) except that 10 times the usual concentration of phosphate salts was employed.

Cultures were prepared for ultraviolet irradiation as follows: about 10^7 spores of the strain to be investigated were inoculated into 5 ml of casamino acid broth in tubes. These cultures were incubated with shaking at 37° until the optical density at 650 mμ had reached 0.500 to 0.600. Three milliliters of such a culture was pipetted into a pressed bottom petri dish and irradiated, with vigorous shaking, for the indicated number of seconds at a distance of 14 inches from a GE germicidal lamp (15 watts). Two milliliters of the ir-

radiated suspension was added to tubes containing 5 ml of fresh casamino acids medium and the cultures were further incubated as described in the text. At selected intervals, cultures were filtered through sintered glass filters or chilled immediately and centrifuged in the cold to remove the cells. The filtrate or supernatant fluid was then assayed for phospholipase, edema factor, and bacteriophage particles.

The bacteriophage assay methods were those described by Thorne (10). The best indicator strain for the phage was *B. cereus*, strain 569. Phospholipase was assayed by the egg yolk broth method reported by Molnar (5) except that incubation was continued overnight at 37°. The phospholipase titer is the reciprocal of the highest dilution showing a positive reaction. The guinea pig skin test for toxin was performed as described by Thorne *et al.* (9). Twofold serial dilutions of the sample in gelatin phosphate are injected intracutaneously on either side of the vertebral column. After 18 to 24 hours, edema appears at the site of injection of toxin-containing samples. The highest dilution at which an edema reaction can be palpated constitutes the titer of the sample tested. Mitomycin C was dissolved in sterile 0.1 *M* phosphate buffer, pH 7.4, to a concentration of 100 μg/ml and refrigerated until needed. Stock solutions of actinomycin D (1 mg/ml) and 5-fluorouracil deoxyriboside (5-FUDR) (1 mg/ml) were prepared in 0.1 *M* phosphate buffer, pH 7.4, and sterilized by filtration through a sintered glass filter. These solutions were stored at 4° until used.

Results

1. *Release of Edema Factor (EF) and Phospholipase (PL) by U.V. Treated or Mitomycin C Exposed Cells*

The production of phospholipase and toxin (edema factor) during post-irradiation incubation of cells is presented in Fig. 1. Both EF and PL appear in

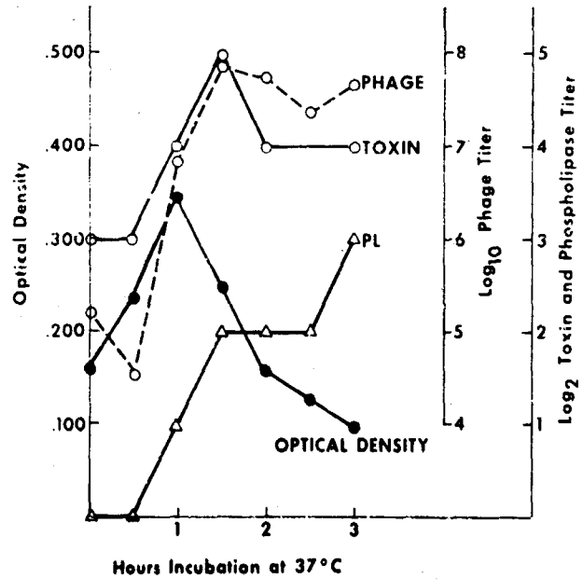

FIG. 1. Toxin, phospholipase, and bacteriophage release from strain 6464 following ultraviolet irradiation. Incubation was on shaker.

the culture filtrate concurrently with an increased number of mature phage particles. Subsequently, the titer of these two activities rose to a maximum and in some cases declined after this peak. In this experiment there is a fall in toxin titer whereas the phospholipase titer remains high although in other experiments there was a small drop in phospholipase titer as well. Losses in lecithinase activity in cultures of *B. cereus* have been attributed to physical effects of shaking, probably surface denaturation (4).

The excellent induction produced by mitomycin C is presented in Fig. 2. In this case the culture was grown up to an optical density of 0.200 and mitomycin C was added so that the final concentration was 0.1 μg/ml. Immediately and every 30 minutes thereafter a tube was filtered and the filtrate assayed for EF, PL, and bacteriophage particles. The results which we obtained with mitomycin C are very similar to those of Otsugi *et al.* (6) wherein a lysogenic *Escherichia coli* strain could be induced by exposure to mitomycin C.

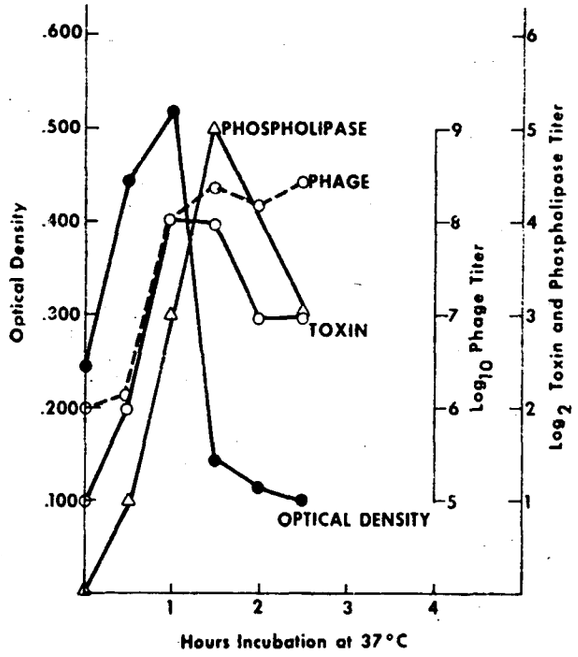

FIG. 2. Toxin, phospholipase, and bacteriophage release following mitomycin C induction of strain 6464. All incubation was on the shaker.

2. The Effects of Agitation on Irradiated Cultures

General experience in this laboratory has indicated that there was better production of phospholipase and edema factor in static cultures as compared to cultures incubated on a shaker. Accordingly the release of PL and EF by ultraviolet irradiated cells was determined by postirradiation incubation in the stationary or shaken condition. The results presented in Table I indicate that higher titers of both PL and EF are produced under static incubation. The increases in the titer of these two activities preceded the measurable decline in optical density. An increase in the number of bacteriophage particles was also noted.

TABLE I

Production of toxin and phospholipase by shaken and static cultures of ultraviolet-induced 6464

Minutes after irradiation	Shaken				Static			
	Phage titer	Optical density	Toxin	PL	Phage titer	Optical density	Toxin	PL
0	1.6×10^{5}	.160	8	0	3.8×10^{5}	.145	2	0
30	3.2×10^{4}	.235	8	0	3.7×10^{5}	.185	8	2
60	6.8×10^{6}	.345	16	2	7.7×10^{5}	.220	32	4
90	6.6×10^{7}	.250	32	4	1.7×10^{6}	.270	64	4
120	6.1×10^{7}	.160	16	4	9.9×10^{6}	.310	64	8
150	2.5×10^{7}	.130	8	4	4.7×10^{7}	.305	32	8
180	4.9×10^{7}	.100	16	4	1.1×10^{8}	.210	64	8

NOTE: Cells of strain 6464 grown on shaker to optical density of 0.600 and irradiated for 20 seconds. Postirradiation incubation conducted at 37 °C as specified in table. Toxin and phospholipase (PL) titers are reciprocals of the highest dilution of filtrate exhibiting the respective activity.

3. *Spontaneous Release of Phage, Edema Factor, and Phospholipase*

The foregoing data illustrate the coincidence between cell lysis and the release of phospholipase and edema factor. Subsequently, cultures unirradiated but receiving otherwise identical treatment were examined to determine the relationship between phage, phospholipase, and edema factor in the absence of u.v. induction. The data in Table II show that during growth of unirradiated cells, there is a release of some toxin (edema factor) and phospholipase accompanied by an increase in phage titer. The titer of these agents decreases upon further incubation, probably attributable to surface denaturation (edema factor and phospholipase) and readsorption in the case of bacteriophage.

TABLE II

Toxin, bacteriophage, and phospholipase production by unirradiated cultures of *B. cereus* strain 6464

Minutes after dilution	O.D.	Toxin	Phospholipase	Phage/ml
0	.220	2	2	7×10^{3}
30	.410	2	4	9.4×10^{3}
60	.630	8	16	4.5×10^{4}
90	.785	8	32	3.8×10^{5}
120	.850	2	32	1.6×10^{4}
150	.900	2	16	3.8×10^{3}
180	.950	2	—	8×10^{2}

NOTE: Cells grown on shaker to optical density of 0.600 and diluted as in irradiation experiments. Postdilution incubation on shaker at 37 °C. Toxin and phospholipase titers are reciprocals of the highest dilution of filtrate exhibiting the respective activity.

4. *Effect of Temperature of Incubation*

In a number of enteric organisms it is known that, after infection with a temperate bacteriophage, the initial establishment of the lysogenic condition is favored by relatively low temperature (20–25°) whereas vegetative replication yielding cell lysis is favored by somewhat higher temperatures (35–37°). The data in Table III show that the amount of PL, EF, and bacteriophage liberated decreased as temperature of postirradiation incubation was lowered.

TABLE III

Production of phage, toxin, and phospholipase by cells of strain 6464 at various postirradiation incubation temperatures

Minutes after irradiation	Postirradiation incubation temperature											
	37 °C				30 °C				26 °C			
	O.D.	Toxin	PL	Phage	O.D.	Toxin	PL	Phage	O.D.	Toxin	PL	Phage
0	.160	8	0	1.6×10^{5}	.180	2	2	2.4×10^{5}	.170	8	4	5×10^{6}
30	.235	8	0	3.2×10^{4}	.245	4	—	2.7×10^{5}	.220	8	4	1.5×10^{5}
60	.345	16	2	6.8×10^{6}	.290	4	4	1.7×10^{5}	.250	8	4	2.7×10^{5}
90	.250	32	4	6.6×10^{7}	.415	4	8	2.8×10^{6}	.290	8	4	8×10^{6}
120	.160	16	4	6.1×10^{7}	.460	16	8	8.5×10^{6}	.360	16	8	3.2×10^{6}
150	.130	8	4	2.5×10^{7}	.430	16	8	1.8×10^{7}	.420	8	8	3.5×10^{6}
180	.100	16	4	4.9×10^{7}	.400	16	16	1.9×10^{7}	.465	8	16	2.4×10^{6}

NOTE: Cells grown on shaker to optical density of 0.600 and irradiated for 20 seconds. Postirradiation incubation on shaker at indicated temperature. Toxin and phospholipase (PL) titers are reciprocals of the highest dilution of filtrate exhibiting the respective activity.

TABLE IV

Inhibition by chloramphenicol (CMP), actinomycin (ACTI-D), or 5-fluorodeoxyuridine (5-FUDR) of the mitomycin C-induced lysis of the cured strain

	Optical densities at 650 mμ								
	Expt. 1			Expt. 2			Expt. 3		
Minutes after induction	No additions	MITO C alone	MITO C + CMP	No additions	MITO C alone	MITO C + ACTI-D	No additions	MITO C alone	MITO C + 5-FUDR
0	.250	.250	.250	.220	.220	.220	.220	.200	.200
30	.460	.440	.360	.405	.405	.320	.350	.350	.270
60	.620	.585	.435	.580	.550	.340	.570	.520	.320
90	.770	.620	.510	.770	.630	.370	.730	.485	.370
120	.875	.500	.580	.890	.575	.380	.920	.300	.420
150	.970	.315	.640	.950	.335	.410	.975	.180	.470
180	1.00	.225	.710	.950	.200	.420	.975	.140	.520
210	1.00	.180	.740	.950	.165	.450	—	—	—

NOTE: Mitomycin C added to final concentration of 0.2 μg/ml. Final concentration of other inhibitors were chloramphenicol, 10 μg/ml; actinomycin D, 0.5 μg/ml and 5-FUDR, 5.0 μ/ml.

It should be noted in the data of Table III that each temperature of post-irradiation incubation represents a separate experiment. Therefore, the base-line titers at zero time are different for each temperature; however, the change in titer following irradiation is the important consideration.

5. Isolation of a Clone Cured of the Bacteriophage

The foregoing evidence indicated a relation between multiplication of the temperate bacteriophage and the elaboration of edema factor and phospholipase. It was of considerable interest to investigate the response to irradiation and the release of edema factor and phospholipase by a culture of cells cured of the functional prophage carried by the parent strain. Consequently, spores of the parent strain were incubated in casamino acids medium until the optical density showed about a 50% increase. At this time, the culture was irradiated with ultraviolet light for varying lengths of time. After each interval of irradiation, an aliquot was removed and suitably diluted and plated by being spread on the surface of nutrient agar plates. Irradiation for 240 seconds allowed only 0.15% survival of the original plating centers. A large number of clones of surviving cells was picked and these were screened to detect sensitivity to the phage produced by the parent. One such clone, referred to as the cured strain, was found to be sensitive to the bacteriophage from the parent strain and to give an efficiency of plating 100-fold greater than the indicator strain, B-569, routinely employed. Assessment of ultraviolet induced lysis and edema factor and phospholipase production by the cured strain gave the results presented in Fig. 3. These data show that the strain cured of the complete prophage still possesses an ultraviolet inducible lytic system which liberates edema factor and phospholipase upon cell lysis. Thus the possible critical role of the functional prophage in the formation of either edema factor or phospholipase is excluded; however, this experiment demonstrates anew that cell lysis leads to the elaboration into the medium of these two activities.

6. Effect of Inhibitors on Induced Lysis of the Cured Strain Using Mitomycin C as the Inducer

In an attempt to learn the nature of the inducible lytic response in the cured strain, the action of inhibitors of protein, RNA, and DNA synthesis was determined. Cells grown in the casamino acids medium were induced by the addition of 0.2 μg of mitomycin C per ml. Immediately thereafter suitable concentrations of these inhibitors were added and the response as measured by optical density changes was determined. The results presented in Table IV show that lysis of induced cells could be completely prevented by addition of either chloramphenicol or actinomycin D or 5-fluorodeoxyuridine. Such results indicate that macromolecular synthesis following induction is requisite for the processes leading to cell lysis and that the three vital classes of biological macromolecules, namely protein, RNA, and DNA, are involved in the postinduction lysis (see Discussion).

Discussion

Other studies about the relation between lysogeny and toxinogeny in *Corynebacterium diphtheriae* have shown that classical lysogenic conversion

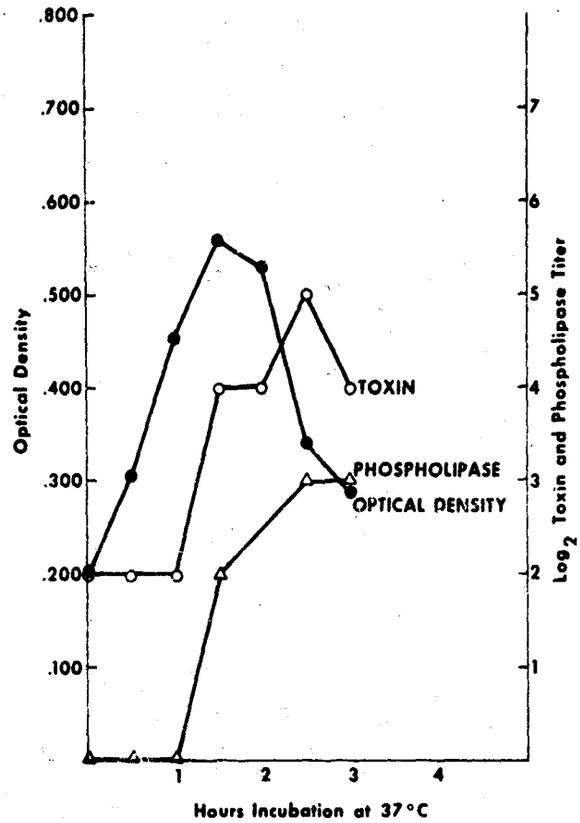

FIG. 3. Toxin and phospholipase release following ultraviolet irradiation of 'cured' clone of strain 6464. All incubation was on shaker.

is not involved in any simple causal relation. Instead, there is ample evidence that induction of lysogenic diphtheria bacilli leads to release of toxin only during and after actual cell lysis by the intracellularly multiplying bacteriophages (2). In the cases reported here, a very similar situation seems to exist. All the evidence presented shows that edema factor and phospholipase are released in conjunction with cell lysis and that this occurs even in the absence of the complete prophage carried by the parent strain. Clearly both the parent strain and the cured strain possess some inducible lytic system whose operation ultimately results in the release of both phospholipase and edema factor into the medium. Indeed, it appears that this is the mechanism of toxinogeny in this strain of *B. cereus* as opposed to a hypothesis which would require that internally produced edema factor and phospholipase diffuse out of the intact cell.

It would be advantageous to be able to study formation of intracellular edema factor produced before cell lysis; however, the lability of this toxic factor to the usual methods of cell extraction has so far prevented this approach. It is not at all certain that intracellular edema factor would be found even with a sufficiently gentle extraction method. This substance (edema

factor) might be present in a latent form in the cell envelope and only upon cell lysis (rupture) would the cell envelope be exposed to intracellular enzymes which could then free the toxic entity. A very similar concept is the mechanism of formation of bradykinin by the action of trypsin on serum proteins (3).

A considerable release of edema factor and phospholipase upon induction of the cured strain is of great interest. Although the complete prophage has been eliminated, there still remains an inducible lytic system which effects the release of the two substances studied here. The results with inhibitors show that the remaining inducible lysis can be prevented by chloramphenicol, actinomycin D, or 5-fluorodeoxyuridine, which prevented the synthesis of protein, RNA, and DNA, respectively. In this regard, the lytic system strongly resembles defective lysogeny or bacteriocinogeny, which are similar phenomena. However, attempts to detect a hypothetical second phage by plating filtered lysates of the initially cured strain on a wide variety of *B. cereus* strains and *Bacillus* species were unsuccessful. Cells of the cured strain were subjected to the usual curing procedure as outlined in the results section in the hope that a clone could be found which was cured of the hypothetical second phage and would be an indicator or which was no longer inducible to lysis by ultraviolet light or mitomycin C but such a clone could not be found. Electron microscopy of lysates of the cured strain to detect morphologically distinct but defective phages was not attempted. Although the fact that an inhibitor of DNA synthesis prevented induced lysis of the cured strain strongly suggests that phage replication precedes lysis, no direct evidence of double lysogeny of the parent strain 6464 has been obtained.

Acknowledgments

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